Title: Cloning pBad-RFP on pSB1C3

Goal: To test the leakiness of the pBad promoter by assembling a pBad-RFP-1C3 plasmid
Date range: May – June, 2015
Members that worked on project: Naomi Iverson

Results:
-Successfully assembled pBad-RFP onto a 1C3 backbone.
-Ran trails with different arabinose concentrations to test leakiness of pBad promoter once plasmid was assembled.

New part number: BBa_K1718001

Lengths of parts:
BBa_I0500 = 1210bp
PCRed RBS-RFP = 12bp + 706bp = 718bp
pSBIC3 = 2070bp

Total part size: 3998bp

Transformation:

- Pulled BBa_I0500 (pBad) and BBa_J04450 (RFP) from iGEM kit.
- 1 ul of plasmid from iGEM kit into 40ul of 5 alpha competent cells.
- Let cells sit with plasmid on ice for 30 minutes.
- Heat shock at 42°C for 45 seconds.
- Put on ice for 5 minutes.
- Treat cells with 950ul of SOC.
- Put in incubator at 37°C for 1 to 2 hours.
- Spread 100ul of cells onto chloramphenicol resistance plates (170ng/ul concentration)

Overnight:

- Make 1 overnight of 5mL of LB with 25ul chloramphenicol.

Mini-prep:

- BBa_I0500 concentration: 121.2ng/ul.
- BBa_J04450 concentration: 249ng/ul.

PCR of BBa_J04450:

- Purpose: Get the RFP gene off from J04450 and to add the RBS site in front of the gene.
- PCR polymerase: Q5 HF 2x Master Mix.
- Primers used: GEM027 Forward and Reverse.

Reagent	25 ul reaction	Concentration
2x Q5	12.25 ul	1 x
10 uM F. Primer	1.25 ul	0.5 uM
10 uM R. Primer	1.25 ul	0.5 uM
DNA (249.0 ng/ul)	1 ul	0.249 ng
Q5 polymerase	0.25 ul	0.02 U/ul
Water	9.0 ul	

Reaction conditions:

Step	**Temp (C)**	**Time**
I. Denature	98	30 s.
35 cycles	98	10 s
	64	15 s
	72	20 s
Final extension	72	2 min.

- Annealing temp: 64°C
- Extension time: 20 s

PCR clean-up:

- Elution volume: 30ul
- Concentration: 66.0 ng/ul

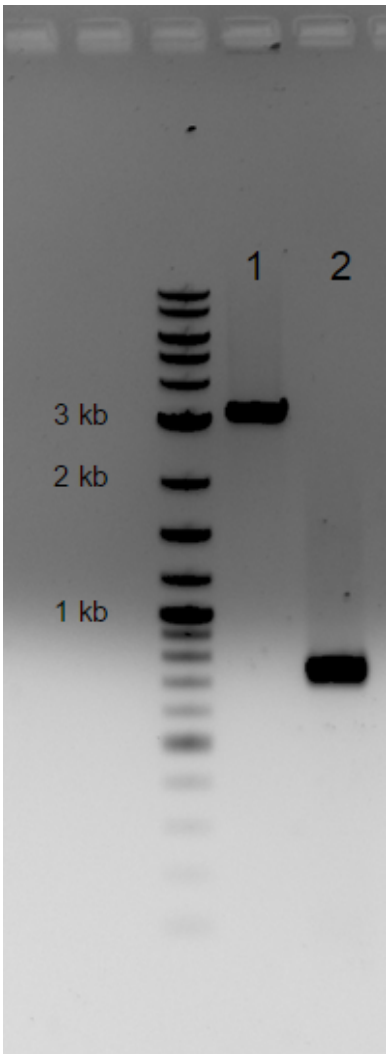
Gel of pBad and RBS-RFP:

- Latter: 2-log
- Gel concentration: 1%

RBS-RFP and pBad
(July 1st, 2015)

Lane 1: I0500 (pBad on 1C3) ~3280bp
(1210bp from pBad + 2070bp from 1C3)
Digested with SpeI and PstI

Lane 2: J04450 (RBS-RFP) ~718bp
Digested with XbaI and PstI



Digestion of BBa_I0500 (pBad):

- Purpose: digest at S and P cut sites to make sticky ends for ligation with the RBS-RFP PCR product from BBa_J04450
- Enzymes used: SpeI-HF and PstI-HF

Reagent	**50 ul reaction**	**Concentration**
Cutsmart Buffer	5 ul	1 x
SpeI-HF	1 ul	10 units
PstI-HF	1 ul	10 units
DNA (121.2 ng/ul)	4 ul	500 ng
Water	39 ul	

Digestion of RBS-RFP PCR product from BBa_J04450:

- Purpose: digest at X and P cut sites to make sticky ends for ligation with BBa_I0500 (pBad)
- Enzymes used: XbaI-HF and PstI-HF

Reagent	**50 ul reaction**	**Concentration**
Cutsmart Buffer	5 ul	1 x
XbaI-HF	1 ul	10 units
PstI-HF	1 ul	10 units
DNA (66.0 ng/ul)	7.58 ul	500 ng
Water	35.42 ul	

Ligation:
Vector = pBad-1C3
Insert = RBS-RFP

Component	**20 ul reaction**	**Concentration/amount**
10XT4 DNA Ligase Buffer	2 ul	1 x
Vector DNA (2070 bp)	36.3 ng	0.027 pmol
Insert DNA (1928 bp)	100 ng	0.08 pmol
Nuclease-free water	To 20 ul	500 ng

Let reaction run for 12 hours at 16°C then heat shock at 65°C for 10 minutes.

Then perform transformation by using previously described transformation procedure. Only difference: instead of using 1 ul of DNA, put 10 ul of DNA into a tube of 40 ul of alpha 5 competent cells.

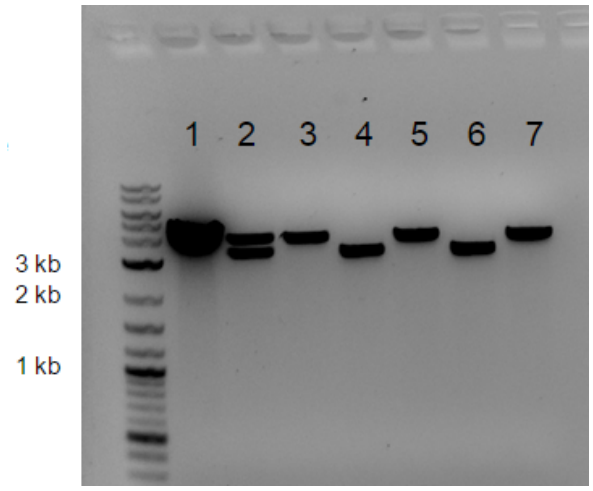
pBad-RBS-RFP Ligated Final Product
(July 2, 2015)

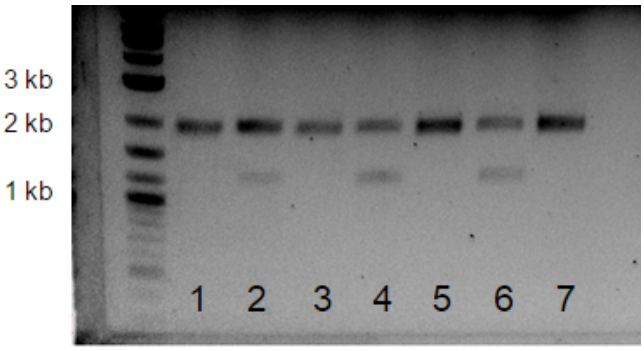
Lanes 1-7 in top gel: pBad+RFP plasmid (each lane from a different colony on plate from transformation) digested with just **EcoRI**. Note: in comparing these lanes with bottom gel, we have concluded that lanes 3, 5 and 7 contain the correct constructs we're looking for.

Lanes 1-7 in bottom gel: pBad+RFP plasmid (each lane here corresponds to the lane in the top gel) digested with both **EcoRI and PstI**. Note: in comparing these lanes with the top gel, we have concluded that lanes 3, 5 and 7 contain the correct constructs we're looking for because the insert (pBad+RFP = 1928bp) and vector (1C3 = 2070bp) are roughly the same size, therefore there is a double band in lanes 3, 5, and 7 in this gel. Lanes 2, 4, and 6 are missing RFP based off from the insert size.

pBad ~ 1210bp
RFP ~ 718bpt
1C3 backbone ~2070bp

Total length ~3998bp



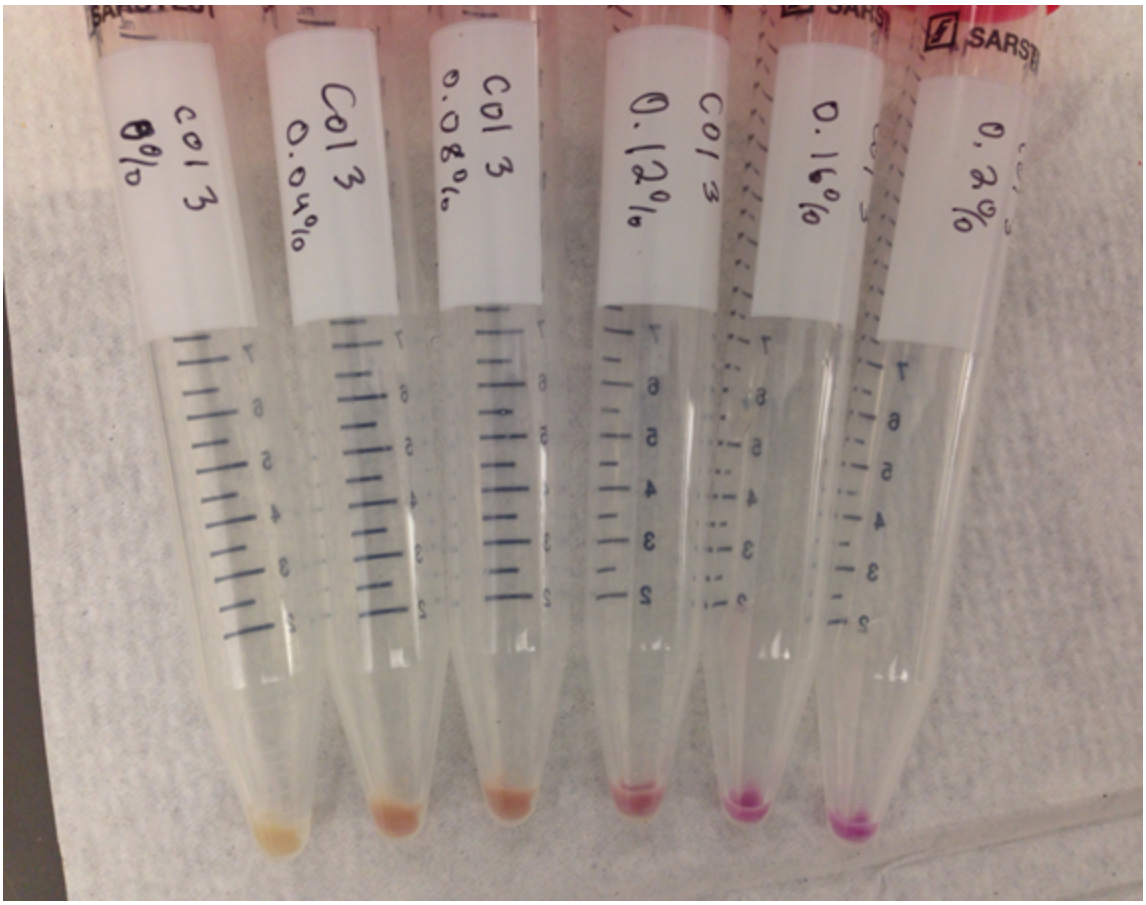


Initial characterization:
The promoter, pBad, turns on in the presence of arabinose and in doing so, allows RFP to be transcribed. Therefore, once arabinose is present, our cells will fluoresce red.

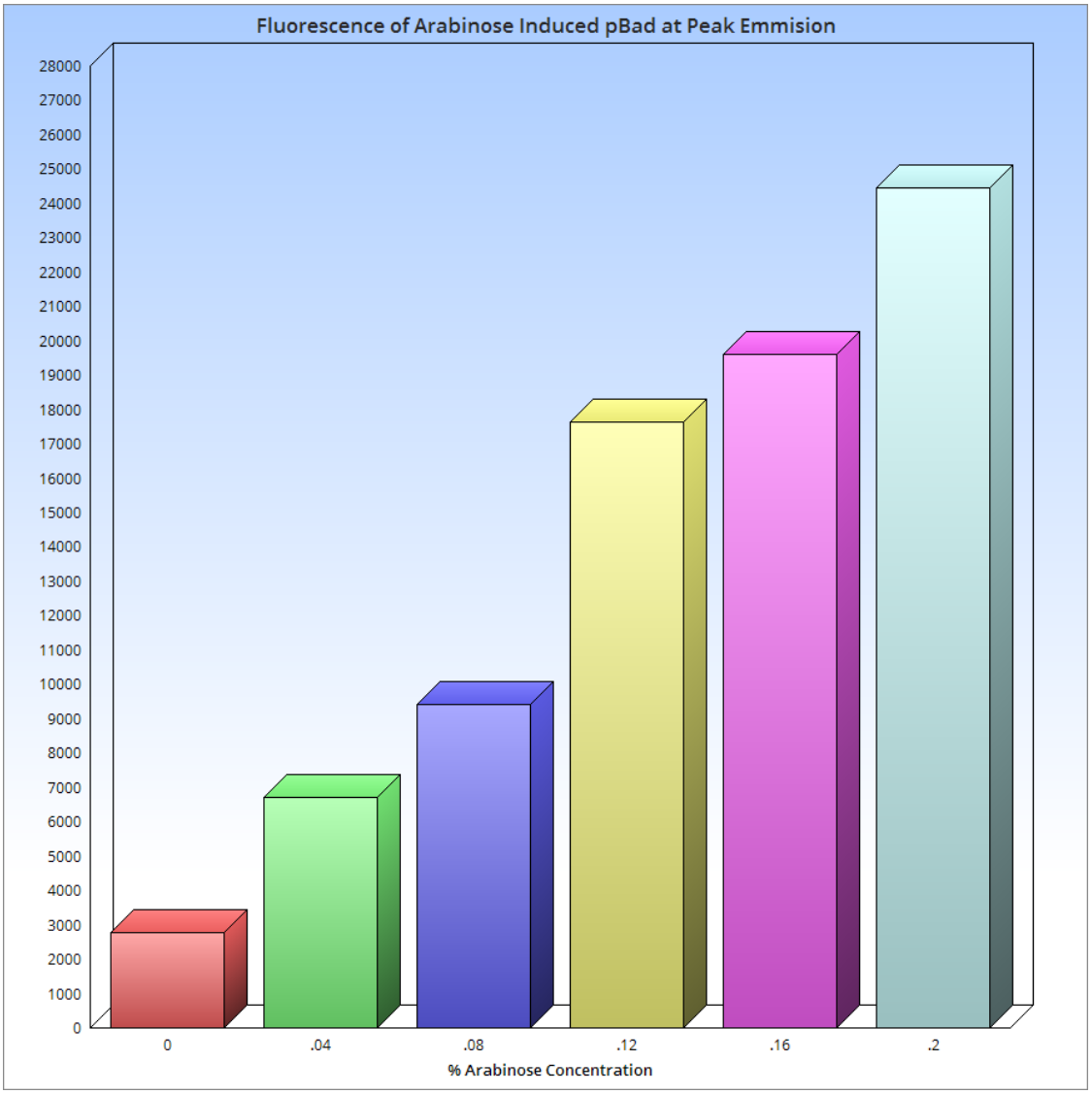
The amount that our cells fluoresce depends on the amount of arabinose. To further characterize this, we grew up overnights with varying concentrations of arabinose from 0% to 0.2% by 0.04% increments. The cells grew for 16 hours then were spun at 6800 rpm. The brightness of the pellets were then compared by eye.

Initially, we documented with pictures to illustrate the change in fluorescence and we observed a relatively smooth increase in brightness as the arabinose concentration increased. By eye, we don't observe any red in the 0% arabinose tube.

Varied concentrations of arabinose from 0% to 0.2% by 0.04% increments (7/14/15):



Then we used flow cytometry to more accurately characterize the fluorescence:



As illustrated in the flow cytometry characterization, we do see some cells fluorescing even when no arabinose is present, therefore the pBad promoter is leaky.

Our device simply requires a human to monitor the cells and therefore since the human eye cannot pick up any red in the 0% arabinose, our promoter works fine for its intended purposes. However to further reduce the leakiness, we have attacked the issue from various angles such as putting the construct on a lower copy plasmid, therefore reducing the effects of leakiness.

Sequence:

pBad

RBS

RFP

1C3

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